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Maternal Exposure of BALB/c Mice to Indoor NO_2 and Allergic Asthma Syndrome in Offspring at Adulthood with Evaluation of DNA Methylation Associated Th2 Polarization

Huifeng Yue,[1] Wei Yan,[1] Xiaotong Ji,[1] Rui Gao,[1] Juan Ma,[2] Ziyu Rao,[2] Guangke Li,[1] and Nan Sang[1]

[1]College of Environment and Resource, Research Center of Environment and Health, Shanxi University, Taiyuan, Shanxi, People's Republic of China
[2]State Key Laboratory of Environmental Chemistry and Ecotoxicology, Research Center for Eco-Environmental Sciences, Chinese Academy of Sciences, Beijing, People's Republic of China

Background: Fetal stress has been proposed to be associated with diseases in both children and adults. Epidemiological studies suggest that maternal exposure to nitrogen dioxide (NO_2) contributes to increased morbidity and mortality of offspring with allergic asthma later in life.

Objectives: We aimed to test whether maternal NO_2 exposure causes allergic asthma-related consequences in offspring absent any subsequent lung provocation and whether this exposure enhances the likelihood of developing allergic asthma or the intensity of developed allergic airway disease following postnatal allergic sensitization and challenge. In addition, if such consequences and enhancements occurred, we sought to determine the mechanism(s) of these responses.

Methods: Pregnant BALB/c mice were exposed to either NO_2 (2.5 ppm, 5 h/day) or air daily throughout the gestation period. Offspring were sacrificed on postnatal days (PNDs) 1, 7, 14, 21, and 42, and remaining offspring were sensitized by ovalbumin (OVA) injection followed by OVA aerosol challenge during postnatal wk 7–9. We analyzed the lung histopathology, inflammatory cell infiltration, airway hyper-responsiveness (AHR), immune responses, and gene methylation under different treatment conditions.

Results: Maternal exposure to NO_2 caused a striking increase in inflammatory cell infiltration and the release of type 2 cytokines in the lungs of offspring at PNDs 1 and 7; however, these alterations were reversed during postnatal development. Following OVA sensitization and challenge, the exposure enhanced the levels of allergic asthma-characterized OVA-immunoglobulin (Ig) E, AHR, and airway inflammation in adult offspring. Importantly, differentiation of T-helper (Th) 2 cells and demethylation of the interleukin-4 (*IL4*) gene occurred during the process.

Conclusions: Maternal exposure to indoor environmental NO_2 causes allergic asthma-related consequences in offspring absent any subsequent lung provocation and potentiates the symptoms of allergic asthma in adult offspring following postnatal allergic sensitization and challenge; this response is associated with the Th2-based immune response and DNA methylation of the *IL4* gene. https://doi.org/10.1289/EHP685

Introduction

Nitrogen dioxide (NO_2) is a gaseous pollutant found in both outdoor and indoor environments. It is emitted via automobile exhaust, fossil fuel combustion, and power plant operations, all of which compose the majority sources of outdoor exposures, whereas indoor exposures are commonly due to emissions from cooking gas, heating systems, and sites with increased occupational exposures such as garages and ferries (Ezratty et al. 2014; Gaffin et al. 2014). Importantly, indoor air NO_2 pollution is likely more serious than outdoor pollution (Ezratty et al. 2014) with maximum concentrations indoors reaching peaks of 2–4 ppm (WHO 2010). NO_2 exposure has been associated with asthma symptoms and the decline of lung function, and recent evidence has shown that early life exposure to NO_2 is a potential stimulus for the early genetic encoding of asthma and allergic responses (Clark et al. 2010; Deng et al. 2016b; Khreis et al. 2017). In addition, such exposure has been considered as an important determinant in the later development of asthma in childhood and adulthood (ATSDR 2014; Deng et al. 2015, 2016a). A study conducted by Mortimer et al. (2008) in California indicated that prenatal NO_2 exposure led to a decrease in the forced vital capacity (FVC) and forced expiratory volume in one second (FEV1) among children. Another study reported that nitrogen oxide (NO_x, primarily NO_2) exposure during the second trimester induced impaired lung development and worsened asthma symptoms in the offspring (Padula et al. 2010). Recently, it has been reported that maternal exposure to the air pollutant NO_2 during pregnancy, especially during certain trimesters, was associated with an increased risk of the development of asthma in children (Deng et al. 2016b).

Allergic asthma is a chronic inflammatory disorder of the lung characterized by airway hyper-responsiveness (AHR), the recruitment of eosinophils and increased serum immunoglobulin E (IgE) levels. The dysregulation of specific T lymphocytes and their associated cytokines plays an important role in the etiology of allergic asthma (Lambrecht and Hammad 2015; Holgate 2012; Lloyd and Hessel 2010). The onset of allergic asthma is characterized by the increased infiltration of naïve $CD4^+$ T lymphocytes into the bronchial mucosa. When these $CD4^+$ T cells are sensitized by an allergen, they become activated and differentiate into proallergic T-helper (Th) 2 cells instead of the counterregulatory Th1 cells (Hwang et al. 2017; Muehling et al. 2017). The incidence of allergic asthma has risen steadily over the past 20 years and currently represents a significant health and financial burden. The reasons for this increase remain unclear, although a link between air pollution and allergic sensitization may be correlated with the prevalence of allergic asthma. Experimental studies have provided evidence of a biological basis for gases such as NO_2 and particulate air pollutants as risk factors for allergic sensitization as indicated by enhanced IgE production (Weir et al. 2013). NO_2 inhalation can augment the degree of allergic airway inflammation and prolong allergen-induced airway hyper-responsiveness (AHR) in rodent models of asthma (Poynter et al. 2006). Similarly, epidemiological studies of two birth cohorts in Germany and Sweden have shown a positive association between

Address correspondence to N. Sang, College of Environment and Resource, Shanxi University, Taiyuan, Shanxi 030006, PR China. Telephone: 86-351-7011932. Email: sangnan@sxu.edu.cn

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air pollution and allergic sensitization (Morgenstern et al. 2008; Nordling et al. 2008). Increased levels of NO_2 have been consistently associated with an increased prevalence of allergic sensitization (Weir et al. 2013); however, information regarding the effects of NO_2 exposure during early life, especially fetal development stages, and in combination with postnatal exposure to allergens, on the manifestation of allergic asthma later in life has rarely been assessed, and the lack of detailed experimental evidence warrants further investigation.

In our study, we treated pregnant BALB/c mice with NO_2 and subjected postnatal offspring to selective allergic sensitizations and subsequent challenges to address the following: *a*) whether allergic asthma-related consequences manifest in juvenile offspring in the absence of subsequent lung provocation; *b*) what responses, if any, develop in adult offspring to postnatal allergic sensitization and challenge; and *c*) how maternal NO_2 exposure causes postnatal respiratory and immune abnormalities following allergen sensitization and challenge.

Methods

Animal Protocol

BALB/c mice (7–8 wk of age) were purchased from the Beijing Vital River Laboratory Animal Technology Co., Ltd. and maintained on a 12-h light/dark cycle with unrestricted access to ovalbumin (OVA)-free food and water, except when kept in the dynamic exposure chamber. All animal care and experiments were approved by the Institutional Animal Care and Use Committee of Shanxi University. The animals were treated humanely and with regard to the alleviation of pain and suffering. At 9–10 wk of age, 156 female mice were mated (one male and two females per cage), and 101 plug-positive mice were considered pregnant. The day on which a vaginal plug was observed was defined as gestation day 0 (GD0).

NO_2 Exposure and OVA Sensitization/Challenge

Plug-positive mice were randomly divided into an NO_2 inhalation group containing 51 animals and a control group of 50 animals. For NO_2 exposure, plug-positive animals, in individually housed wire cages, were exposed to NO_2 (2.5 ppm, 5 h/day) daily in a dynamic exposure chamber starting at GD0, and the treatment lasted throughout the gestation period. The NO_2 gas was diluted with air at the intake port of the exposure chamber to achieve the desired concentration, and the mixed gas was uniformly distributed throughout the entire chamber through two perforated gas-dispersion plates. One plate was placed at the intake port, and the other was placed on the gas outlet connected to an aspirator pump. The NO_2 concentration in the exposure chamber was measured using a real-time NO_2 monitor (FIX550-NO2-A, WANDI Technology Co.), and the exhaust gas was absorbed by an alkali absorption device. Correspondingly, the control plug-positive animals were continually exposed to air in the other exposure chamber using the same protocol. During the exposure period, the animals and their cages were placed in the exposure chamber, and the mice were free to move around in the cages but were denied access to water and food. Pregnant mice were housed in separate cages until the offspring could be weaned, and none of the offspring were exposed to NO_2 after birth. At postnatal day (PND) 21, the offspring from the different litters were housed together (no more than 10 animals/cage), and males and females were separated. The offspring from the NO_2 inhalation group and from the vehicle control group were sacrificed on PND1, 7, 14, 21, and 42, and the lungs were dissected for histological examination (two females and two males per time point); flow cytometry analysis (fluorescence-activated cell sorting (FACS), five females and five males per time point); enzyme-linked immunosorbent assay (ELISA) detection (10 females and 10 males on PND1, eight females and eight males on PND7, and five females and five males on PND14, 21, and 42); and methylation status analysis (three mice per time point).

The remaining offspring from the NO_2 inhalation and vehicle control groups were administered either OVA [80 μg OVA and 1.3 mg colloid $Al(OH)_3$; Sigma Chemical Co.], colloid $Al(OH)_3$ (1.3 mg; Sigma Chemical Co.) or sterilized saline via a single intraperitoneal (i.p.) injection at 7 and 8 wk of age. Then, the OVA or saline challenge was followed by inhalational exposure to either 1% OVA or saline at 9 wk of age (30 min/day for 7 d) using an ultrasonic nebulizer until the offspring were sacrificed at 10 wk. Following the $Al(OH)_3$ or OVA sensitization and OVA challenge, the offspring from mothers exposed to air or NO_2 were designated the AAO [Air + $Al(OH)_3$ + OVA] ($n = 40$), AOO (Air + OVA + OVA) ($n = 41$), NAO [NO_2 + $Al(OH)_3$ + OVA] ($n = 40$), and NOO (NO_2 + OVA + OVA) ($n = 43$) groups, and the corresponding control groups subjected to saline treatment were classified as the ASS (Air + saline + saline) ($n = 40$) and NSS (NO_2 + saline + saline) ($n = 42$) groups. After allergen sensitization and challenge, the offspring from the various groups were sacrificed, and the lungs were dissected for FACS analysis (five females and five males), histological examination (two females and two males), the measurement of lung function (five females and five males), ELISA (five females and five males), and the assessment of methylation status (four mice). The pre- and postnatal treatment protocol is shown in Figure 1.

Measurement of AHR

At 24 h after the final treatment, the AHR was assayed as previously described using an AniRes2005 Lung Function System (version 3.5; Bestlab Technology Co.) according to the manufacturer's instructions (You et al. 2014). Briefly, mice were anesthetized with 50 mg/kg pentobarbital sodium (Beijing Solarbio Technology Co., Ltd.) and connected to a computer-controlled ventilator via the tracheal cannula. The time of expiration/inspiration and the respiratory rate were preset at 1.5:1 and 90/min, respectively. The resistance of the lung (R_L), resistance of expiration (Re), and respiratory dynamic compliance (Cdyn) were recorded to evaluate the reaction of mice to a methacholine chloride [Acetyl-β-methylcholine chloride (MCH) (Sigma)] gradient (0.025, 0.05, 0.1, and 0.2 mg/kg body weight); this compound was injected into the jugular vein at 5-min intervals using a fine needle.

FACS Analysis

Mouse lungs were carefully perfused, excised, diced, and incubated with collagenase type IA (0.5 mg/mL; Sigma-Aldrich Corp.) and type IV bovine pancreatic DNase (20 μg/mL; Sigma-Aldrich Corp.) for 45 min at 37°C in Hank's Balanced Salt Solution (HBSS) containing 5% fetal bovine serum (FBS). The digested lung tissue was passed through a 40-μM cell strainer to form a single-cell suspension, and TruStain fcX (antimouse CD16/32) was added to the suspension to block the Fc receptors on the cells. To determine the percentage of macrophages, eosinophils, neutrophils, and lymphocytes in the lung tissue, the cells were stained for extracellular antigens using specific antibodies according to standard instructions (Table 1) (Barletta et al. 2012; Ford et al. 2012; Sharma et al. 2016). The percentage of $CD4^+$ interleukin (IL)-4^+ (Th2) cells was measured after the suspensions were washed with PBS before surface staining. To determine the intracellular expression of IL-4, cells were first treated

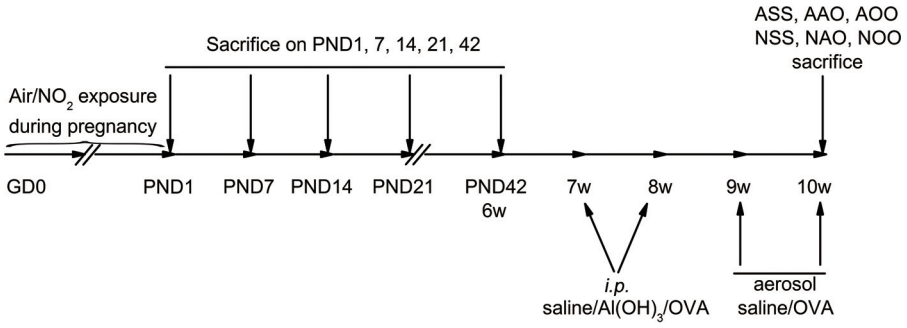

Figure 1. Experimental timeline for prenatal and postnatal treatments and sacrifice indicating the times of maternal NO_2 exposure and postnatal OVA sensitization and OVA challenges. Times are expressed relative to the birth date of the offspring. Note: GD, gestation day; PND, postnatal day; w, week; i.p., intraperitoneal.

with a cell stimulation and protein transport inhibition cocktail containing PMA, Ionomycin, Brefeldin A, and Monensin (500x, eBioscience, Thermo Fisher Scientific (China) Co., Ltd.) for 4 h followed by surface staining (PerCP-conjugated antimouse CD45, APC-conjugated antimouse CD3, and FITC-conjugated antimouse CD4 [BioLegend Inc.,]). The cells were fixed with 2% PFA, permeabilized with 0.5% Triton X-100 and stained with a PE-conjugated antimouse IL-4 antibody (eBioscience, Thermo Fisher Scientific (China) Co., Ltd.) (Jeon et al. 2014; Wang et al. 2013). All the experiments set isotype control. The FACS analysis was performed using a BD LSRII flow cytometer (BD Biosciences) according to standard protocols as previously described. The details of the gating strategy are shown in Figure S1.

Determination of Cytokines

After the mice were sacrificed, the lungs were harvested, and 60 mg of tissue per animal was homogenized in 300 μL PBS containing 1% (wt/vol) Triton X-100 (Beijing Solarbio Technology Co., Ltd.) and pepstatin A, leupeptin, and aprotinin (all at 20 ng/ml, pH 7.4; Beijing Solarbio Technology Co., Ltd.) and incubated on ice for 30 min as previously described (Wieland et al. 2006). The homogenate was then centrifuged (4°C, 1,500 *g*, 15 min), and the supernatant was assayed for IL-4, IL-13, and interferon (IFN)-γ using the appropriate ELISA reagent kits (R&D Systems, Inc.) according to the manufacturer's instructions.

Histological Examination

After a lung lavage and the removal of the right lung lobes, the left lung lobe was intratracheally perfused with 10% neutral buffered formalin at a constant pressure of 30 cm of fixative. After 1 h, the trachea was ligated, and the inflated left lung lobe was immersed in a large volume of the same fixative for at least 24 h. The following day, the tissue was embedded in paraffin, and segments (5- to 6-μm-thickness) were prepared for staining with hematoxylin and eosin (H&E), Alcian blue (pH 2.5), and Periodic acid Schiff's (AB/PAS) reagent or Masson's trichrome (MT); five sections were used per stain (Li et al. 2009).

Table 1. The antibodies used for staining antigens by FACS analysis.

Number	Antigen	Fluorochrome	Company
1	CD3	APC	Biolegend
2	CD4	FITC	Biolegend
3	CD45	Percp	Biolegend
4	CD11b	PE/cy7	Biolegend
5	Gr-1	PE	Biolegend
6	I-A/I-E	FITC	Biolegend
7	F4/80	PE	Biolegend
8	Siglect-f	eflour 660	eBioscience
9	IL-4	PE	eBioscience

Total IgE and OVA-Specific IgE Detection

At 24 h after the last OVA aerosol exposure, blood specimens were collected using evacuated tubes (Taizhou Tianyi Medical Devices Co., Ltd.) and incubated at room temperature (RT, 20–25°C) for 30 min. The blood samples were then centrifuged at 1,000 *g* for 10 min at RT, and the resulting supernatant (serum) was stored at −80°C until further analysis. The levels of total IgE and OVA-specific IgE in the serum were measured using ELISA reagent kits (Mouse IgE ELISA MAX™ Deluxe Sets and LEGEND MAX™ and Mouse OVA-Specific IgE ELISA Kit with Pre-coated Plates; BioLegend Inc.) according to the manufacturer's instructions.

Analysis of the Methylation Status of the IL4 and IL13 Promoters

The methylation status of the *IL4* and *IL13* promoters was measured as previously described (Zhou et al. 2016). Genomic DNA was extracted using a Tissue DNA Kit (Omega Bio-tek, Inc.) and treated with bisulfite according to the EZ DNA Methylation-Gold™ Kit instruction manual (Zymo Research). The *IL4* and *IL13* promoter sequences were amplified from the isolated DNA using touchdown PCR, extracted from an agarose gel and incorporated into the Puc18-T vector [Sangon Biotech (Shanghai) Co., Ltd.] for TA cloning and sequencing by Sangon Biotech [Sangon Biotech (Shanghai) Co., Ltd.]. The PCR conditions were as follows: 98°C for 4 min; 20 cycles of 45 s at 94°C, 45 s at 66°C (with a 0.5°C decrement per cycle), and 1 min at 72°C; 20 cycles of 45 s at 94°C, 45 s at 56°C and 1 min at 72°C; and a final extension at 72°C for 8 min. The prediction of the CpG islands and the designation of the BSP primers for the *IL4* core promoter sequence (−566 to −311 and −41 to +244) and the *IL13* sequence (−1915 to −1554 and −355 to −81) were performed by Sangon Biotech. The BSP primer sequences were as follows: CpG region 1 of *IL4*, forward 5'-TTGTAAGATTAGTTGGTTTAGGATG-3', and reverse 5'-TTTCAACATAAAAAATTACACCATA-3'; CpG region 2 of *IL4*, forward 5'-GTTAGTATTGTATTGTTAGTATTTTTTGAT-3', and reverse 5'-ATCTCTTAAACTTTATCCCTAATCCTA-3'; CpG island 1 of *IL13*, forward 5'-TTTATTGTAGYGGGGYGGT-3', and reverse 5'-ACCTTAAACRCTACATAAATAAATCA-3'; and CpG region 2 of *IL13*, forward 5'-GGTTAGTATTGGGTTGGTTGTTTAG-3', and reverse 5'-CCTAAACTACTAACTTATAACCTTAACCTA-3'. In detail, five individual clones from one mouse, a total of 15 clones of three mice from PND1 and PND42, and five individual clones from one mouse and a total of 20 clones of four mice from each treatment were sequenced. In a given sample, the number of the

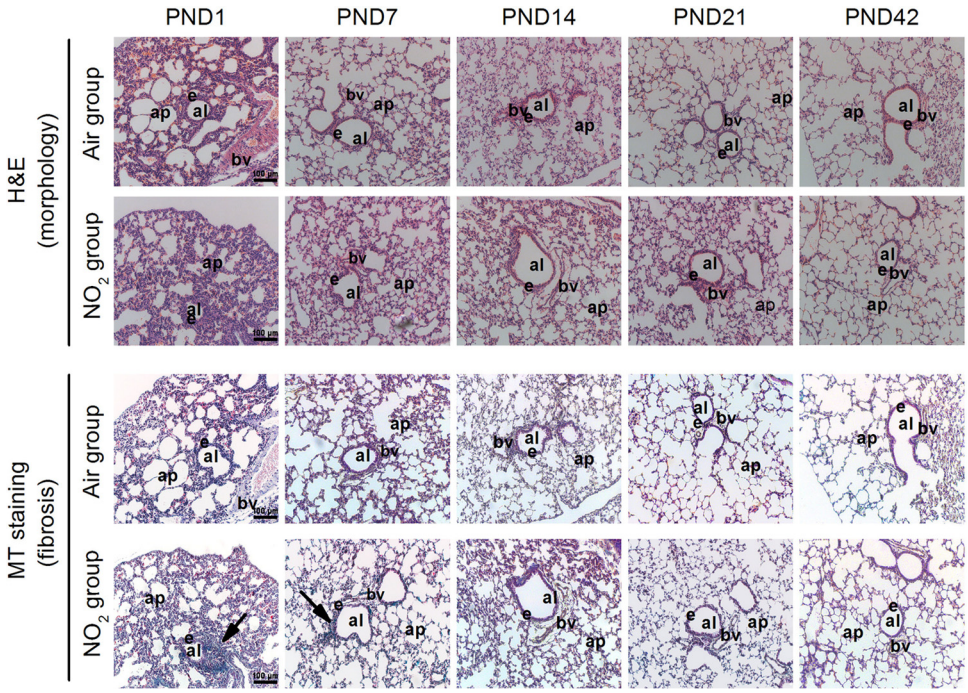

Figure 2. Representative photomicrographs of histology pictures of airway structures in the lungs of offspring at PND1, 7, 14, 21, and 42. H&E staining shows the inflammatory cell infiltration; MT staining shows subepithelial collagen deposition. Note: H&E, hematoxylin and eosin stain; MT, Masson's trichrome stain; al, airway lumen; ap, alveolar parenchyma; bv, blood vessel; e, airway surface epithelium; arrows, sites of fibrosis. Bar = 100 μm.

methylated CpG sites was divided by the total number of detected CpG sites among the five clones to evaluate the methylation percentage of each CpG region for one animal; furthermore, the methylation status of each region within the *IL4* and *IL13* promoters from each treatment group were calculated by averaging the methylation rate of each CpG region from three or four animals (Tang et al. 2012; Zhang et al. 2014). Visualization and analysis of the methylation status of the CpG regions surrounding the *IL4* and *IL13* promoters were conducted using a BiQ Analyzer (http://biq-analyzer.bioinf.mpi-inf.mpg.de/tools/MethylationDiagrams/index.php).

Statistical Analysis

The data are expressed as the mean ± standard error. A one-way analysis of variance (ANOVA) followed by Fisher's least significant difference (LSD) test was used to determine significant differences between all the treatment groups and the control group. A two-tailed Student's *t* test was used to analyze the experimental results between pairs of groups. Significance was defined as $p < 0.05$, and the data analysis and figure generation were conducted using Origin® 8.0 (OrigenLab Corp.).

Results

There were 267 offspring from 39 litters in the control group comprising 139 female and 128 male offspring, and there were 286 offspring from 42 litters in the NO_2 inhalation group comprising 160 female and 126 male offspring. There were no significant differences in either the delivery rate or litter size between the NO_2- and air-exposed dams; additionally, none of the offspring died due to exposure.

Maternal NO_2 Exposure, Airway Inflammation, and Th2 Polarization in Juvenile Offspring

We first investigated whether allergic asthma-related consequences arose in the offspring subjected to maternal NO_2 exposure from GD0 to birth in the absence of subsequent lung provocation. As shown in Figure 2, maternal NO_2 inhalation caused inflammatory cell infiltration in the peribronchial and perivascular areas (H&E staining) and peribronchial collagen deposition (MT staining) in PND1 and seven mice, although no obvious mucous cell metaplasia (AB-PAS staining) was observed at any time point (data not shown). Interestingly, these pathological abnormalities were attenuated during postnatal development and were nearly eliminated at PND21 and 42. Consistent with this, we observed that the total number of cells in the lungs of the offspring at PND1 increased slightly by 1.52-fold in comparison with that in the corresponding control pups, suggesting that maternal NO_2 exposure mildly promotes the recruitment of cells. To quantify the potential inflammatory response, we further analyzed the leukocyte subtypes by differentiating the recruited cell populations using FACS. Figure 3 indicates that maternal NO_2 exposure resulted in increased recruitment of macrophages, eosinophils, and lymphocytes, with eosinophils presenting the most obvious increase in the lungs of offspring at PND1, 7 and 14. However, no significant increase in the number of neutrophils was observed at any time point. As expected, the total number of cells began to decline at PND7, and the recruitment of macrophages and lymphocytes was gradually eliminated by PND14; however, eosinophils were still detected.

Cellular inflammation of the airways with eosinophils is a characteristic feature of allergic asthma (Zissler et al. 2016; Loutsios et al. 2014). Coupled with airway inflammation, T cells in the airways in human and animal models of allergic asthma present cytokine profiles characteristic of Th2 cells (Zissler et al. 2016; Singh et al. 2011). Based on the above findings, we hypothesized that maternal NO_2 exposure may differentially induce naïve $CD4^+$ T-cell polarization toward Th2 cells instead of Th1 cells. To test this hypothesis, we analyzed the expression of the type 2 cytokines IL-4 and IL-13 and the type 1 cytokine IFN-γ. As shown in Figure 4, maternal NO_2 exposure elevated the IL-4 levels and suppressed IFN-γ expression at PND1 and

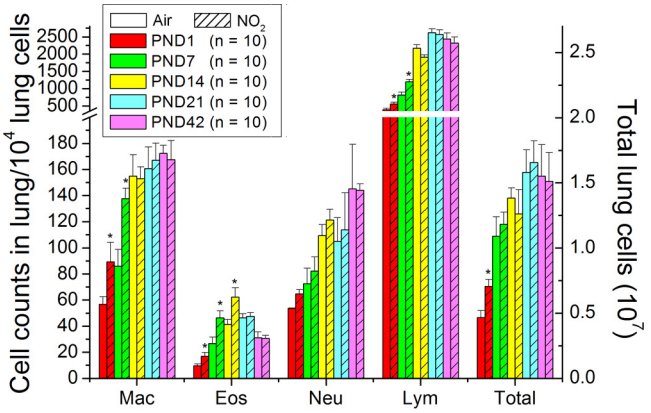

Figure 3. Effects of maternal NO_2 exposure on the differentiation of inflammatory cells in the lungs of offspring at PND1, 7, 14, 21, and 42. The values are expressed as the mean ± SE. $*p < 0.05$ compared with the air exposure group. Note: Mac, macrophages; Eos, eosinophils; Neu, neutrophils; Lym, lymphocytes.

PND7; moreover, these effects tended to subside at PND14 and even returned to normal levels at PND21 and 42. Additionally, the IL-13 content was statistically increased at PND1 but restored at PND7.

Importantly, we controlled for sex in the statistical analyses and provide these data in the Supplemental Materials (Figure S2). The results indicated that the basic values in male offspring were slightly different from those in female offspring, including the number of eosinophils and the IL-4 level, but the fold changes were equal in the two sexes, suggesting no sex-related significant difference in the inflammatory responses and the expression of the type 2 cytokines IL-4 and IL-13 and the type 1 cytokine. These findings suggest that maternal NO_2 exposure caused allergic asthma-related consequences in the offspring, including airway inflammation and Th2 polarization; however, these effects subsided during postnatal development in the absence of subsequent lung provocation.

Maternal NO_2 Exposure, OVA-Specific IgE Release, AHR Alteration, and Lung Cell Differentiation in Offspring in Response to OVA Sensitization and Challenge

Next, we sought to clarify the effects (if any) of maternal NO_2 exposure on offspring subjected to allergic sensitization and challenge. To address this issue, we combined maternal NO_2 exposure with postnatal exposure to OVA to elicit an allergic response via sensitization and subsequent challenge with OVA. The OVA sensitization and subsequent challenge apparently increased the levels of total IgE regardless of the type of maternal inhalational exposure, and the levels in the AOO and NOO groups were 3.86- and 3.94-fold higher than the values in the ASS and NSS groups and 1.44- and 1.53-fold higher than the values in the AAO and NAO groups, respectively. No significant difference was observed between the NOO and AOO groups. OVA-specific IgE was undetectable in the ASS and NSS groups but apparently increased in the AOO and NOO groups, suggesting that allergic asthma was successfully established in this study. Importantly, the levels of OVA-specific IgE in the NOO group significantly increased to 1.96- and 2.58-fold of those in the AOO and NAO groups, respectively (Figures 5A and B).

As predicted, OVA sensitization and challenge significantly increased AHR in the offspring regardless of maternal exposure to air or NO_2 (Figure 5C). For all the groups, the Re values increased with increasing doses of MCH, and at 0.2 mg/kg MCH, the Re values in the AOO and NOO groups were 1.36- and 1.96-fold higher than those in the ASS and NSS groups and 1.38- and 1.86-fold higher than those in the AAO and NAO groups, respectively. R_L showed the same trend as Re, whereas Cdyn showed an opposing trend to that of Re. Furthermore, the AHR in the NOO group was significantly higher than that in the AOO and NAO groups. However, maternal NO_2 exposure without OVA sensitization and challenge did not induce apparent AHR. AHR directly reflects changes in the airway wall structure. Common structural changes in the airway include thickening of the subbasement membrane, excessive mucus secretion, subepithelial fibrosis, inflammatory cell infiltration, and extracellular matrix deposition in the subepithelial layer (You et al. 2014). Consistent with this, we observed that OVA-sensitized and subsequently challenged mice presented inflammatory cell infiltration in the peribronchial and perivascular areas, mucous cell metaplasia, and peribronchial collagen deposition. Importantly, these alterations in the offspring from the NOO group were more profound than those in the AOO and NAO groups (Figure 6).

Next, we quantified the inflammatory cell differentiation. Figure 7 shows that the OVA sensitization and subsequent challenge significantly recruited more total cells, macrophages, eosinophils, and Th2 cells into the lung in the absence or presence of maternal NO_2 exposure, and the number of eosinophils in the AOO and NOO groups was 2.34- and 4.00-fold greater than that in the ASS and NSS groups and 2.11- and 2.90-fold greater than

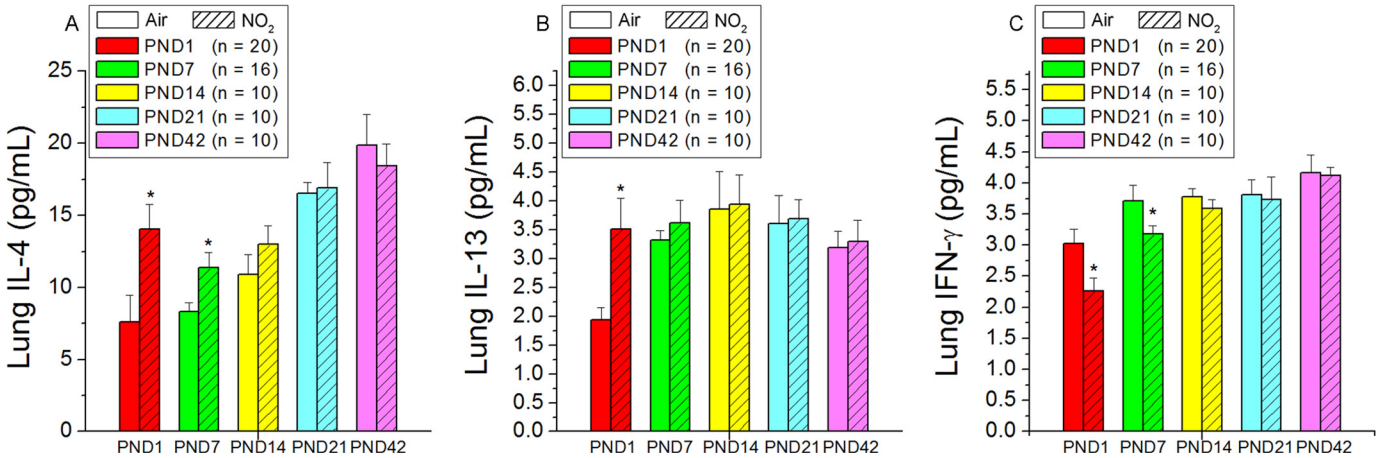

Figure 4. Effects of maternal NO_2 exposure on the levels of the (*A*) type 2 cytokines IL-4 and (*B*) IL-13 and (*C*) the type 1 cytokine IFN-γ in the lungs of offspring at PND1, 7, 14, 21, and 42. The values are expressed as the mean ± SE. $*p < 0.05$ compared with the air exposure group.

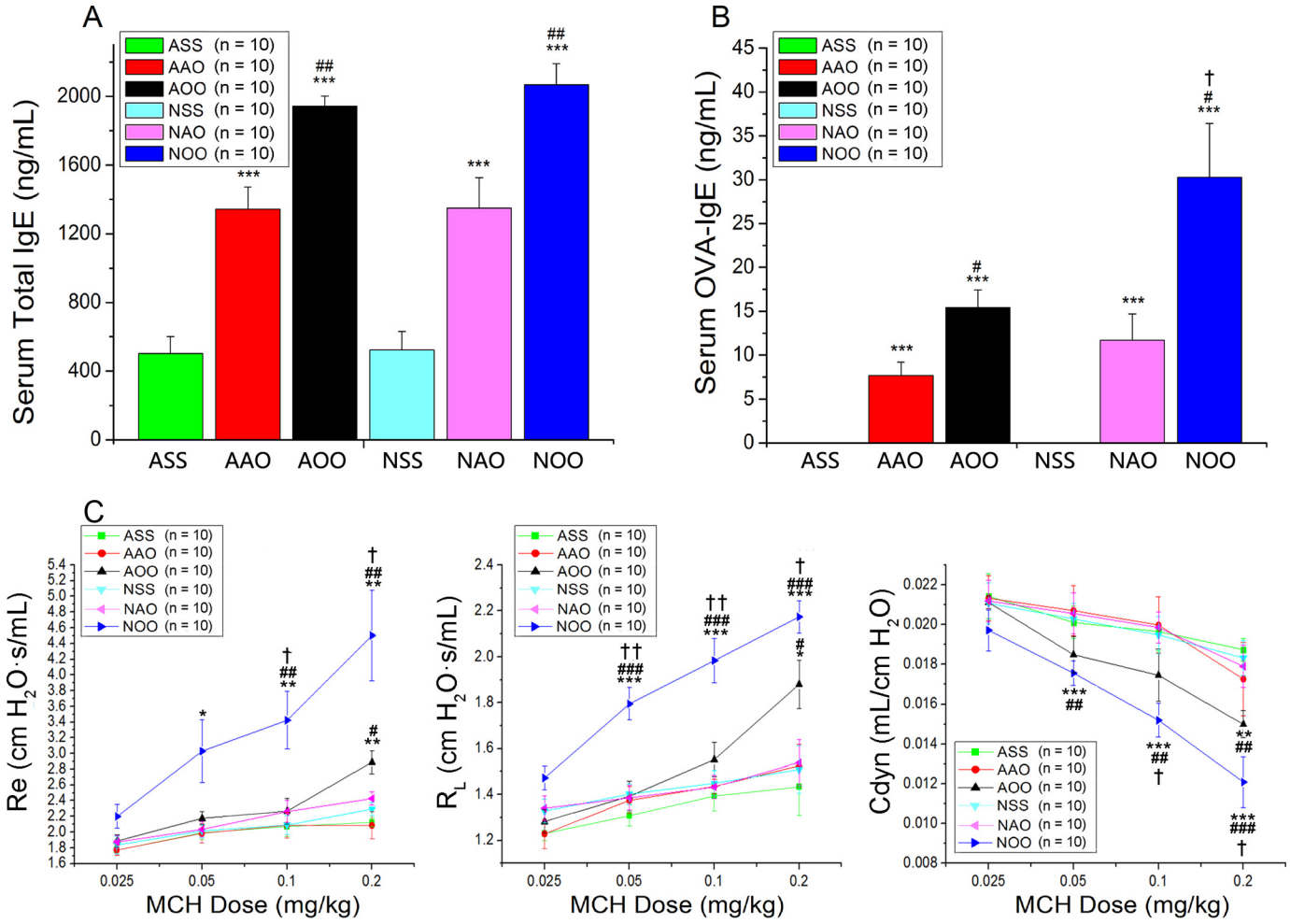

Figure 5. Effects of maternal NO_2 exposure on the symptoms of allergic asthma in offspring subjected to OVA sensitization and challenge. (*A*) Total IgE in the serum of offspring from the different treatment groups. (*B*) OVA-specific IgE in the serum of offspring from the different treatment groups. (*C*) AHR measurements of offspring from the different treatment groups. The values are recorded as the mean ± SE. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ compared with the saline groups. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ compared with the $Al(OH)_3$ groups. $\dagger p < 0.05$, $\dagger\dagger p < 0.01$ compared with the AOO group.

that in the AAO and NAO groups, respectively. Additionally, the number of macrophages in the AOO and NOO groups was 1.73- and 2.84-fold higher than that in the ASS and NSS groups and 1.66- and 2.52-fold higher than that in the AAO and NAO groups, respectively. Regarding Th2 cells, the number in the AOO and NOO groups was 2.01- and 3.04-fold higher than that in the ASS and NSS groups and 1.78- and 3.20-fold higher than that in the AAO and NAO groups, respectively. Moreover, the numbers of eosinophils, macrophages, and Th2 cells in the NOO group were 1.75-, 1.64- and 1.78- fold higher than those in the AOO group, respectively. Interestingly, no significant increases in the neutrophil count were observed in any of the groups. These results suggested that maternal NO_2 exposure enhanced the allergic asthma responses in offspring when combined with postnatal exposure to antigen.

Maternal NO_2 Exposure and Th2 Polarization in Offspring following OVA Sensitization and Challenge

To elucidate the primary immune cell response underlying how maternal NO_2 exposure enhanced symptoms of allergic asthma to OVA sensitization and challenge, we measured the levels of the type 2 and type 1 cytokines. As shown in Figures 8, the levels of IL-4 and IL-13 in OVA-sensitized and subsequently challenged offspring were significantly elevated in the lungs of the offspring, regardless of maternal exposure to air or NO_2. The IL-4 levels in the AOO and the NOO groups were 1.85- and 2.42-fold higher than those in the ASS and NSS groups and 1.63- and 2.16-fold higher than levels in the AAO and NAO groups, respectively. The trend for IL-13 expression was similar to that for IL-4 expression. Importantly, the levels of IL-4 and IL-13 in the NOO group were 1.34- and 1.61-fold higher than those in the AOO group, respectively. In contrast, the IFN-γ levels were significantly decreased in OVA-sensitized and subsequently challenged offspring regardless of maternal exposure to air or NO_2, and the values in the AOO and the NOO groups were 0.72- and 0.64-fold of those in the ASS and NSS groups and 0.80- and 0.67-fold of those in the AAO and NAO groups, respectively. In particular, the content of IFN-γ in the NOO group was 0.82-fold of that in the AOO group. These findings imply that the unbalanced differentiation of naïve $CD4^+$ T cells into Th2 cells instead of Th1 cells plays an important role in the maternal NO_2 exposure-mediated enhancement of allergic asthma in offspring when combined with postnatal exposure to OVA sensitization and challenge.

We also controlled for sex differences in the FACS analysis (five females and five males), lung function measurements (five females and five males), and ELISA detection (five females and five males). As shown in Figure S3, the basic values in the male

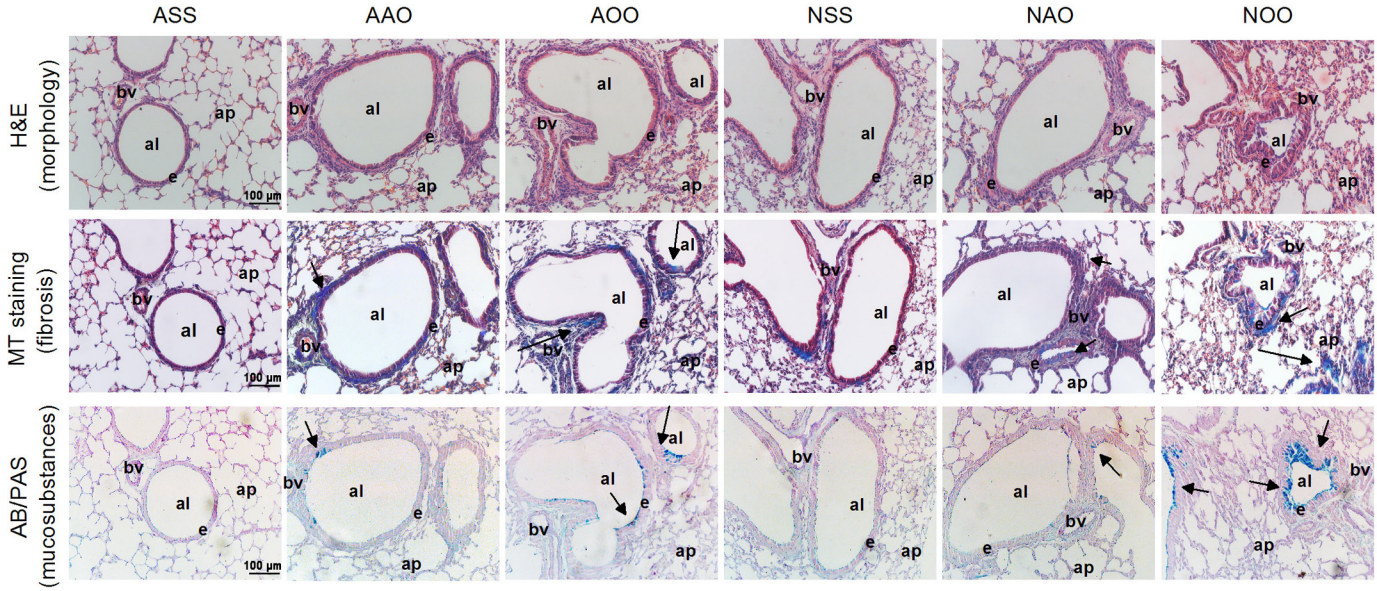

Figure 6. Representative photomicrographs of histology images of airway structures in the lungs of offspring subjected to OVA sensitization and challenge. H&E staining shows the inflammatory cell infiltration; MT staining shows subepithelial collagen deposition; AB/PAS staining shows mucous cells. Note: H&E, hematoxylin and eosin stain; AB/PAS, Alcian blue and Periodic acid Schiff double stain; MT, Masson's trichrome stain; al, airway lumen; ap, alveolar parenchyma; bv, blood vessel; e, airway surface epithelium; arrows, sites of fibrosis and mucous cell metaplasia. Bar = 100 μm.

offspring were slightly different from those of the female offspring, including the AHR, number of eosinophils, OVA-IgE, IL-4, and so on, but the fold changes between the two sexes were equal. These findings suggest no significantly sex-related influence on enhancing the intensity of allergic asthma.

IL4 Promoter Demethylation in Offspring Subjected to Maternal NO_2 Inhalation and Postnatal OVA Sensitization and Challenge

Increasing evidence shows that epigenetic mechanisms are involved in regulating T-cell differentiation, cytokine expression, allergic sensitization, and allergic asthma development (Harb and Renz 2015; Lovinsky-Desir and Miller 2012). Importantly, the methylation status of several CpG sites at the *IL4* and *IL13* genes during Th1/Th2 differentiation and T-cell stimulation has been studied by other researchers (Lee et al. 2015). Therefore, the methylation status of the *IL4* and *IL13* promoters in the lungs of the offspring subjected to different treatment conditions was evaluated. We first investigated whether alterations in gene expression were accompanied by changes in the methylation status of region 1 or region 2 of the *IL4* flanking sequence. Prior to OVA sensitization and subsequent challenge, the methylation status of region 2 in the *IL4* promoter was similar to that of the control group, whereas the methylation status of region 1 was decreased by 20% (Figure 9A) on PND1 but mildly reduced on PND42 (Figure S4A), which is consistent with the observed changes in gene expression. Further, increases in *IL4* expression mediated by OVA sensitization and challenge were associated with reduced promoter methylation of region 1, but the methylation status of region 2 was unchanged (Figure 9B). Interestingly, maternal NO_2 exposure followed by postnatal OVA sensitization and subsequent challenge significantly decreased the methylation rate of region 1 by 9% in comparison with normal air exposure followed by OVA sensitization and subsequent challenge, which is consistent with the change of *IL4* expression. Furthermore, we assessed the methylation status of the *IL13* gene on both CGI (region 1) and nonCGI CpG dinucleotides (region 2). Prior to OVA sensitization and subsequent challenge, the methylation status of region 1 in the *IL13* promoter was similar to that of the control group, but the levels of methylation at region 2 were decreased by 10% (Figure 10A) on PND1 and completely absent on PND42 (Figure S4B), which was consistent with the results of the gene expression analysis. However, OVA sensitization and challenge did not alter the promoter methylation of regions 1 and 2 regardless of maternal NO_2 exposure (Figure 10B), which does not correspond with the changes of *IL13* gene expression. These findings suggest that *IL4* promoter demethylation was associated with Th2 polarization in the offspring in response to maternal NO_2 inhalation and postnatal OVA sensitization and subsequent challenge.

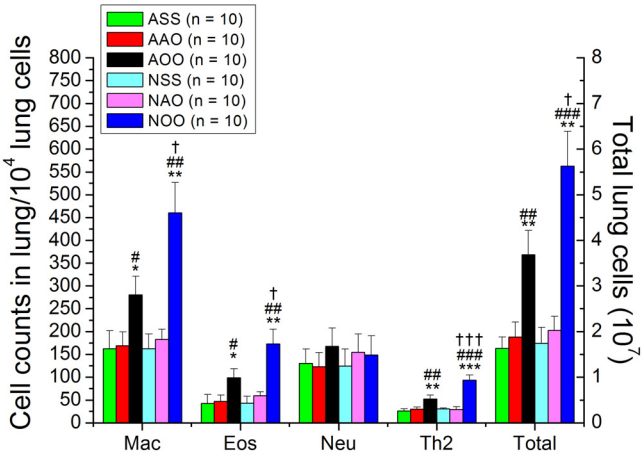

Figure 7. Effects of maternal NO_2 exposure on inflammatory cell differentiation in the lungs of offspring subjected to OVA sensitization and challenge. The values are expressed as the mean ± SE. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in comparison with the saline groups. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ in comparison with the $Al(OH)_3$ groups. $\dagger p < 0.05$, $\dagger\dagger\dagger p < 0.001$ in comparison with the AOO group. Note: Mac, macrophages; Eos, eosinophils; Neu, neutrophils.

Discussion

Although epidemiological studies have suggested that early-life NO_2 exposure may be associated with the increased incidence

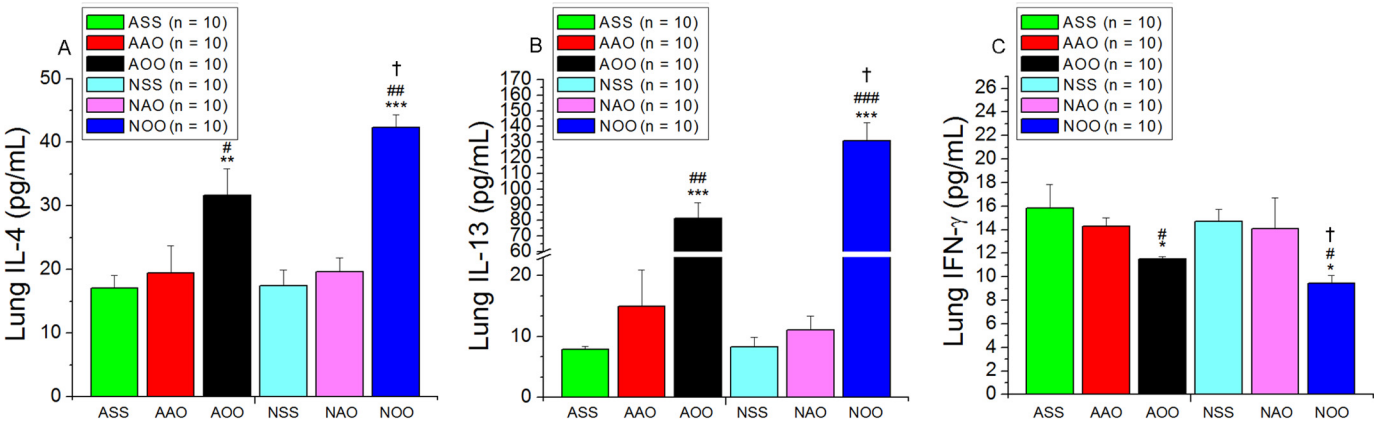

Figure 8. Effects of maternal NO_2 exposure on (*A*) the type 2 cytokines IL-4 and (*B*) IL-13 and (*C*) the type 1 cytokine IFN-γ in the lungs of offspring subjected to OVA sensitization and challenge. The values are expressed as the mean ± SE. $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in comparison with the saline groups. $\#p < 0.05$, $\#\#p < 0.01$, $\#\#\#p < 0.001$ in comparison with the $Al(OH)_3$ groups. $\dagger p < 0.05$ in comparison with the AOO group.

and severity of asthma, the results were controversial (Clark et al. 2010; Deng et al. 2015; Morales et al. 2015; Ranzi et al. 2014). The results of our current study revealed two important points. First, maternal NO_2 exposure caused eosinophilic airway inflammation and an associated release of type 2 cytokines in the lung shortly after birth; second, the effects were almost totally restored to normal physiological levels during postnatal development in the absence of subsequent lung provocation. Similarly, exposure of pregnant dams to lipopolysaccharide (LPS) caused greater pulmonary inflammation in pups at PND 0, 2, 6, and 14, although these differences disappeared by PND 21 (Cao et al. 2009). *In utero* environmental tobacco smoke (ETS) exposure without further lung challenge was observed to have no measurable effect on either pulmonary function or histology in offspring at PND 42 (Penn et al. 2007). Emerging evidence suggests that full maturation of the alveolus occurs during the alveolarization stage (PND 0–14) (Herriges and Morrisey 2014). Following this, we determined the mean linear intercept, alveolar surface area per unit of

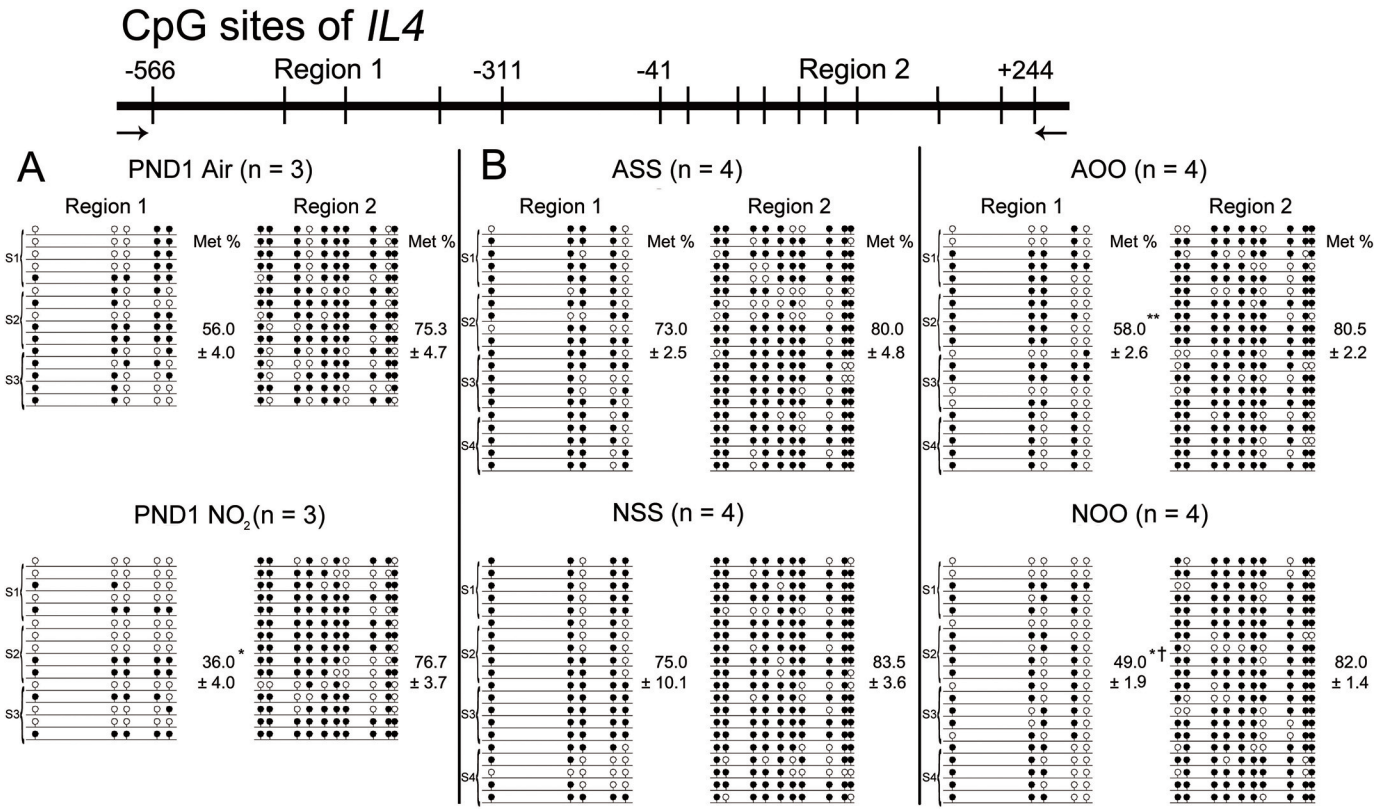

Figure 9. Effects of maternal NO_2 exposure on the methylation status of the *IL4* promoter. (*A*) The methylation status of the *IL4* promoter in the lungs of offspring at PND1. (*B*) The methylation status of the *IL4* promoter in the lungs of offspring from the different treatment groups. Five individual clones from three mice and a total of 15 clones at PND1 were sequenced, and five individual clones from four mice, and a total of 20 clones from the different treatment groups were sequenced. Each row represents an individual clone of the promoter, and a total of five CpG sites on region 1 and 10 CpG sites on region 2 for the *IL4* promoter were analyzed. Each circle represents a CpG site within the promoter; white circles represent unmethylated CpGs, and black circles represent methylated CpGs. Met %, average percent of total CpG methylation. The data are expressed as the mean ± standard error. $*p < 0.05$, $**p < 0.01$ in comparison with air or saline groups. $\dagger p < 0.05$ in comparison with the AOO group. S1, S2, S3, and S4 refer to sample 1, sample 2, sample 3, and sample 4, respectively.

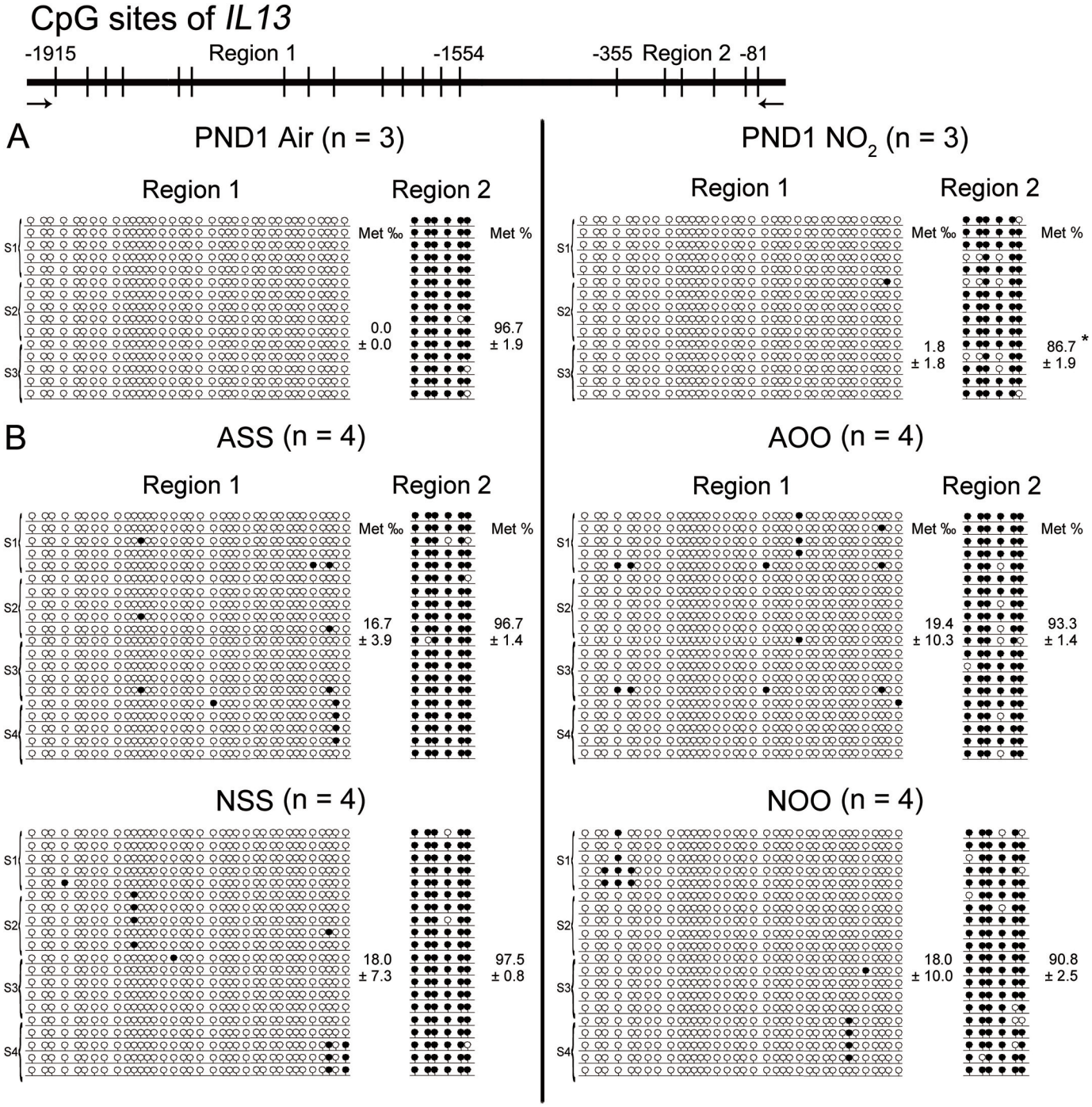

Figure 10. Effect of maternal NO_2 exposure on the methylation status of the *IL13* promoter. (*A*) The methylation status of the *IL13* promoter in the lungs of offspring at PND1. (*B*) The methylation status of the *IL13* promoter in the lungs of offspring from the different treatment groups. Five individual clones from three mice and a total of 15 clones at PND1 were sequenced, and five individual clones from four mice and a total of 20 clones from the different treatment groups were sequenced. Each row represents an individual clone of the promoter, and a total of 36 CpG sites on region 1 and six CpG sites on region 2 for the *IL13* promoter were analyzed. Each circle represents a CpG site within the promoter; white circles represent unmethylated CpGs, and black circles represent methylated CpGs. Met % and Met ‰, average percent of total CpG methylation. Note: The data are expressed as the mean ± standard error. S1, S2, S3, and S4 refer to sample 1, sample 2, sample 3, and sample 4, respectively. $*p < 0.05$ in comparison with air or saline groups.

lung volume, the ratio of lung to body weight, and body weight during postnatal development, and we did not observe any apparent delayed lung development. Thus, the delayed recovery of eosinophilic inflammation in the lung of juvenile offspring might be related to inflammatory cell infiltration, increased collagen deposits in the airways, and increased thickness of the subepithelial basement membrane zone. Although the recruitment of inflammatory cells and associated factors declines during postnatal development, these early abnormalities resemble a maternal NO_2 exposure-induced hyper-responsive airway that becomes more pronounced after additional lung stress or challenge.

To address this implication, we established a mouse model of allergic asthma using OVA sensitization and subsequent challenge and then sequentially evaluated the effects of maternal exposure to NO_2 on allergic manifestations in the offspring. IgE is an important biomarker of asthma that is significantly increased

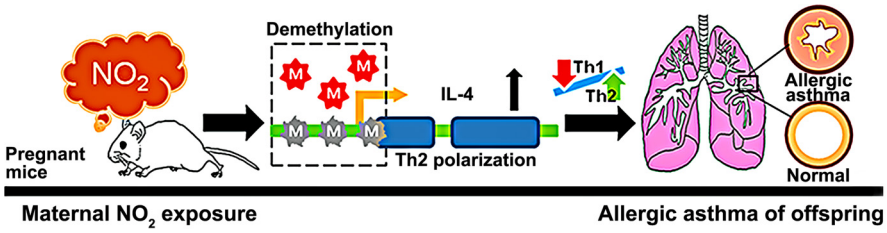

Figure 11. An illustration summarizing the relationship among maternal NO_2 exposure, enhancement of the allergic asthma syndrome in BALB/c offspring, and the associated Th2 polarization and DNA methylation at the *IL4* promoter.

in the serum of individuals with asthma and in animal models of asthma (Froidure et al. 2016). Increased levels of IgE may cause different immune effector cells to release a variety of mediators that promote AHR (Rabe et al. 2011), which is characterized as uncontrolled bronchoconstriction in response to various stimuli. Importantly, exposure of pregnant females to air pollution can lead to elevated IgE levels, long-term deficits in lung function, and even asthma in the offspring (Zacharasiewicz 2016; Peters et al. 2013). AHR in asthma is related to eosinophilic airway inflammation. The eosinophil is a central effector cell that, when localized to an inflamed asthmatic airway, can cause bronchial epithelial damage and airflow obstruction (Kim et al. 2010). Here, we observed that OVA-specific IgE, AHR, and eosinophilic inflammation were all significantly increased in offspring that were sensitized and challenged with OVA and maternal exposed to NO_2 in comparison with offspring that were not exposed to either NO_2 or OVA-sensitization and challenge. It has been reported that a Th2 > Th1 immune disequilibrium contributes to the pathogenesis of allergic diseases (Romagnani 1994). Importantly, prenatal stress exposure increased the susceptibility to allergic AHR and inflammation, which were accompanied by a Th2-dominated immune response (Lee et al. 2015; Penn et al. 2007). Consistent with this observation, the results of the present study show that offspring subjected OVA sensitization and challenge and born to dams exposed to NO_2 during pregnancy exhibited significantly increased IL-4 and IL-13 expression and decreased IFN-γ expression. Th2 cells are activated by various allergens and subsequently initiate allergic immune responses in mild-to-moderate eosinophilic asthma (Vroman et al. 2015). Excessively produced type 2 cytokines (IL-4 and IL-13) cause the infiltration of inflammatory cells into the airway, such as eosinophil influx (Lambrecht and Hammad 2015). In addition, these inflammatory cells produce more cytokines, thus exacerbating airway inflammation and further facilitating allergic responses (Kim et al. 2010). Similarly, prenatal diesel exhaust particle (DEP) exposure enhanced the expression of type 2 cytokines (IL-4, IL-5 and IL-13) and suppressed the expression of type 1 cytokines (IFN-γ) in offspring subjected to OVA immunization and challenge, which resulted in AHR and eosinophilic inflammation (Manners et al. 2014). These findings suggest that maternal NO_2 exposure likely enhances the risk of developing allergic airway diseases or increases the intensity of developed allergic airway disease in offspring subjected to postnatal allergic sensitization and challenge and that the action may be associated with disrupting the balance of Th1/Th2 cell differentiation and the subsequent activation of Th2 immune responses. The underlying mechanisms of NO_2-mediated promotion of allergic asthma in mouse pups remain unclear; however, increasing evidence of the resulting immune responses have been described. NO_2 exposure impaired local bronchial immunity (Guarnieri and Balmes 2014), induced inflammatory cytokine release, and disrupted the balance of Th1/Th2 differentiation (Ji et al. 2015).

Early-life exposure to air pollution can induce epigenetic alterations in gene expression and affect the disease risk for asthma and airway allergies later in life (Martino and Prescott 2011). These alterations frequently involve fluctuations of an aberrant DNA methylation pattern on promoters, accompanied by changes in gene expression; these pattern changes include global hypomethylation and gene-specific hypermethylation or hypomethylation (Lee et al. 2015). Specifically, DNA methylation has been classified as an important modulatory process for the establishment and maintenance of Th2 bias in asthmatic and allergic symptoms (Yang et al. 2015). DNA methylation at the promoter regions of the *IL4* and *IL13* genes are likely associated with an allergic phenotype with Th2 activation (Bégin and Nadeau 2014). Experimental evidence has shown that *in utero* ETS exposure increases the risk of pulmonary inflammation and AHR associated with altered DNA methylation (Lee et al. 2015). Moreover, when female mice (F0) were exposed to *A. fumigatus* during early gestation, the F2 generation developed increased airway eosinophilia and reduced levels of methylation of *IL4* CpG^{-408} and CpG^{-393} (Niedzwiecki et al. 2012). Furthermore, NO_2 exposure during pregnancy was associated with differential DNA methylation in mitochondria- and antioxidant-related genes in the offspring (Gruzieva et al. 2017). In this study, we observed a significant difference in the DNA methylation status at the *IL4* promoter in the lungs of PND1 offspring from the air- and NO_2-exposed dams, but this difference was absent by PND42. Importantly, the DNA hypomethylation status at the *IL4* promoter in the lungs of offspring was statistically increased following postnatal OVA sensitization and subsequent challenge regardless of maternal NO_2 exposure; however, offspring from dams exposed to NO_2 and subjected to the postnatal OVA sensitization and challenge exhibited an enhanced DNA demethylation rate. Additionally, the identified demethylation alterations of the *IL4* gene were consistent with the changes in cytokine expression. In contrast, although the DNA methylation status at the *IL13* promoter in the lung at PND1 showed slight alterations, few methylation sites in the *IL13* promoter were shared among the different treatment groups, which suggests that changes in IL-13 expression were not associated with DNA methylation under these described treatment conditions, and future studies should be conducted to determine how *IL13* is involved in the response to maternal NO_2 exposure. Allergic asthma is defined as bronchial constriction and Th2-dominated airway inflammation following allergen sensitization and subsequent challenge. During this process, IL-4 is an important cytokine predominantly released by Th2 that triggers a humoral immune response toward IgE upregulation and eosinophil accumulation in the airways (Oeser et al. 2015). Consistent with this, Kwon et al. (2008) reported the demethylation of CpG (−80) on the *IL4* promoter of human $CD4^+$ T lymphocytes isolated from patients with allergic asthma following *in vitro* exposure to dust-mite allergens. In response to allergens, the demethylation of type 2 cytokine genes induces a change in the chromatin structure, allowing the DNA to unfold and recruit transcription factors for the immediate expression of type 2 cytokines (Bégin and Nadeau 2014). Our findings provide

initial experimental evidence that maternal NO_2 inhalation-induced *IL4* hypomethylation was associated with Th2 polarization in the offspring, which predisposes the offspring to allergic asthma. Considering that maternal NO_2 exposure increases the number of Th2 cells in the lungs of offspring, regardless of OVA sensitization and challenge, and that IL-4 is primarily produced by Th2, we cannot exclude the notions that *IL4* demethylation is due to the abundance of Th2 cells and that the hypomethylation of *IL4* may facilitate the differentiation of these cells. Furthermore, considering that there are many kinds of cells in the lungs of mice and that the experimental conditions in this reported study did not allow for the specific identification of a cellular subpopulation, the question of whether the altered methylation status is an artifact of differential cellular composition should be addressed in future studies.

Conclusions

Collectively, our findings show that maternal NO_2 exposure resulted in eosinophil-associated airway inflammation and Th2 polarization in the offspring; additionally, these effects were reversed in the absence of subsequent allergen provocation. However, the symptoms of allergy asthma following OVA sensitization and subsequent challenge were enhanced in offspring from dams exposed to NO_2. These effects were associated with a Th2-biased response and DNA demethylation of the *IL4* gene promoter in the offspring (Figure 11).

Acknowledgments

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